



Protein Analysis

SDS polyacrylamide gel electrophoresis

Principle of SDS-PAGE analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules, acquiring a high net negative charge that is proportional to the length of the polypeptide chain. When loaded onto a gel matrix and placed in an electric field, the negatively charged protein molecules migrate towards the positively charged electrode and are separated by a molecular sieving effect. After visualization by a protein-specific staining technique, the size of a protein can be estimated by comparison of its migration distance with that of a standard of known molecular weight. It is also possible to blot the separated proteins onto a positively charged membrane and to probe with protein-specific antibodies in a procedure termed western blotting (see Protocol 7, page 78).

Acrylamide concentration

The concentration of acrylamide used for the gel depends on the size of the proteins to be analyzed. Low acrylamide concentrations are used to separate high molecular weight proteins, while high acrylamide concentrations are used to separate proteins of low molecular weight (Table 2, page 76). Improved resolution of protein bands is achieved by the use of a discontinuous gel system having stacking and separating gel layers.

Preparation of dilute or salt-containing samples for SDS-PAGE

Acid precipitation of proteins (see Protocol 4 below) can be carried out prior to SDS-PAGE analysis in order to concentrate dilute samples or to remove high concentrations of salts that may interfere with the SDS-PAGE procedure.

Protocol 4. TCA precipitation of proteins

Protocol 4

1. Dilute samples to 100 µl; add 100 µl 10% trichloroacetic acid (TCA).
2. Leave on ice for 20 min; centrifuge for 15 min in a microcentrifuge.
3. Wash pellet with 100 µl of ice-cold ethanol, dry, and resuspend in 5x SDS-PAGE sample buffer (see "SDS-PAGE Buffers and Solutions for Analysis of Proteins", page 96). Boil for 7 min at 95°C, and then load samples immediately onto a gel for SDS-PAGE.

Protocol 5. Separation of proteins by SDS-PAGE

Protocol 5

Materials

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| ▶ Gel apparatus and electrophoresis equipment | ▶ TEMED (N,N,N',N'-tetramethylethylenediamine) |
| ▶ 30% acrylamide/0.8% bis-acrylamide stock solution* | ▶ 10% ammonium persulfate |
| ▶ 2.5x separating gel buffer | ▶ Butanol |
| ▶ 5x stacking gel buffer | ▶ 5x electrophoresis buffer |
| | ▶ 5x SDS-PAGE sample buffer |
| | ▶ Protein samples |

Tip Use only high-quality reagents and water for SDS-PAGE. Gel buffers and self-prepared acrylamide/bis-acrylamide stock solutions should be filtered, degassed, and stored at 4°C.

For buffer and reagent compositions, see "SDS-PAGE Buffers and Solutions for Analysis of Proteins", page 96.

* Acrylamide is a potent neurotoxin and is absorbed through the skin. Take appropriate safety measures particularly when weighing solid acrylamide/bisacrylamide, and also when working with the solutions and gels.

protocol continues overleaf



Protocol 5. Continued

Table 2. Compositions and separation properties of SDS-PAGE gels*

Gel acrylamide concentration (%)	Linear range of separation (kDa)	30 % acrylamide/0.8% bis-acrylamide stock solution (ml)	2.5x separating gel buffer (ml)	Distilled water (ml)
15.0	12–43	2.75	2.2	0.55
10.0	16–68	1.83	2.2	1.47
7.5	36–94	1.38	2.2	1.92
5.0	57–212	0.92	2.2	2.38

Adapted from reference 2.

* Volumes given are used for an 8 x 8 or 8 x 10 cm, 1 mm thick minigel, final volume 5.5 ml.

1. Assemble gel plates with spacers according to the manufacturer's instructions.

Tip The plates should be thoroughly cleaned and dried before use.

2. Mark the level to which the separating gel should be poured — a few millimeters below the level where the wells will be formed by the comb.

3. Mix the following in a beaker or similar vessel (for a 12% acrylamide 8 x 8 or 8 x 10 cm, 1 mm thick, minigel).

- ▶ 2.2 ml 30% acrylamide/0.8% bis-acrylamide stock solution
- ▶ 2.2 ml 2.5x separating gel buffer
- ▶ 1.1 ml distilled water
- ▶ 5 µl TEMED

The volumes of acrylamide/bis-acrylamide solution and water should be adjusted according to the percentage acrylamide required (dependent on the size of protein to be separated; see Table 2 above).

Tip The size of the gel apparatus used will determine the volumes of gel solutions necessary.

4. Just before pouring, add 50 µl 10% ammonium persulfate, and mix well. Pour the gel between the assembled gel plates to the level marked in step 2. Overlay with butanol.

Tip Water can be used instead of butanol when using apparatus that may be damaged by the use of butanol — see the manufacturer's instructions.

Tip As soon as ammonium persulfate is added, the gel should be poured quickly before the acrylamide polymerizes.

Tip Prepare ammonium persulfate solution freshly each time it is required.

5. After polymerization is complete (around 20 min), pour off butanol, rinse with water and dry.

Tip Water remaining on the plates can be removed using pieces of filter paper.

6. For the stacking gel, mix the following:

- ▶ 0.28 ml 30% acrylamide/0.8% bis-acrylamide stock solution
- ▶ 0.33 ml 5x stacking gel buffer
- ▶ 1 ml distilled water
- ▶ 2 µl TEMED

7. Just before pouring, add 15 µl 10% ammonium persulfate, and mix well. Pour on top of the separating gel. Insert comb, avoiding introduction of air bubbles.

Tip As soon as ammonium persulfate is added the stacking gel should be poured quickly, before the acrylamide polymerizes.

▶▶▶ protocol continues overleaf



Protocol 5. Continued

Tip With a marker pen, mark and/or number the positions of the wells before removing the comb. This aids loading of samples.

8. After the stacking gel polymerizes (around 10 min), the gel can be placed in the electrophoresis chamber. Fill the chamber with electrophoresis buffer and remove the comb.

9. Before loading, add 1 volume 5x SDS-PAGE sample buffer to 4 volumes of protein sample (i.e., add 2 μ l sample buffer to 8 μ l sample giving a final volume of 10 μ l). Vortex briefly and heat at 95°C for 5 min.

Tip During heating at 95°C, release pressure build up in tubes by briefly opening lids, or piercing a small hole in the lid with a needle. After heating, samples should be briefly centrifuged and vortexed.

10. Load samples and run gel. For electrophoresis conditions refer to the recommendations provided by the manufacturer of the apparatus.

Tip Before loading the samples, rinse out wells with 1x electrophoresis buffer using a suitable syringe and needle.

Tip Load empty wells with 1x SDS-PAGE sample buffer to ensure that sample lanes do not spread out.

Tip Ensure that the electrodes are correctly connected. The proteins will migrate towards the positive (labeled +, usually red) electrode.

Tip Running the gel until the bromophenol blue dye reaches the bottom edge usually gives a satisfactory spread of protein bands.

Visualization of proteins in SDS-PAGE gels

Visualization of protein bands is carried out by incubating the gel with a staining solution. The two most commonly used methods are Coomassie® and silver staining. Silver staining is a more sensitive staining method than Coomassie staining, and is able to detect 2–5 ng protein per band on a gel. Many protocols are available but in order to increase reproducibility, use of a commercially available kit, such as the Bio-Rad® Plus Silver Staining Kit (cat. no.161-0449EDU), is recommended. Silver staining of proteins depends on the reaction of silver with sulfhydryl or carboxyl moieties in proteins and is therefore not quantitative, with some proteins being poorly stained by silver. In addition, after silver staining the protein becomes oxidized and cannot be used for further applications, such as sequencing. Coomassie staining, though less sensitive, is quantitative and Coomassie-stained proteins can be used for downstream applications.

Protocol 6. Coomassie staining

Protocol 6

Materials

- ▶ Coomassie staining solution
- ▶ Destaining solution
- ▶ SDS polyacrylamide gel containing separated proteins (see Protocol 5, page 75)

For buffer and reagent compositions, see “Coomassie Staining Solutions”, page 97.

1. Incubate the gel in Coomassie staining solution for between 30 min and 2 h with gentle shaking. Coomassie Brilliant Blue R reacts nonspecifically with proteins.

2. Gently agitate the stained gel in destaining solution until the background becomes clear (1–2 h).

Tip A folded paper towel placed in the destaining bath will soak up excess stain and allow the re-use of destaining solution.

After destaining the proteins appear as blue bands against a clear gel background. Typically, bands containing 50 ng protein can be visualized.